

Recent Advances in Flavivirus Antiviral Drug Discovery and Vaccine Development

Debashish Ray¹ and Pei-Yong Shi^{*,1,2}

Wadsworth Center¹, New York State Department of Health, and Department of Biomedical Sciences², University at Albany, State University of New York, Albany, New York 12201, USA

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Abstract: Many flaviviruses, including yellow fever virus, dengue virus, Japanese encephalitis virus, tick-borne encephalitis virus, and West Nile virus, are globally important human pathogens. Despite an emergence and resurgence of flavivirus-mediated disease, specific therapies are not yet available; however, significant progress has been made toward the prevention and treatment of flavivirus infections. In this article we review recent advances made in the areas of (i) flavivirus vaccine development, and (ii) ant flavivirus drug discovery reported in literature and patents, and highlight strategies used in these investigations.

Keywords: Flavivirus, vaccine, antiviral, drug discovery, RNA virus, inhibitor.

INTRODUCTIONS

The genus *Flavivirus* consists of over 70 viruses, the majority of which are transmitted by mosquito or ticks [1]. Globally, flaviviruses, including dengue virus (DENV), Japanese encephalitis virus (JEV), tick-borne encephalitis virus (TBEV), yellow fever virus (YFV), West Nile virus (WNV), Murray Valley encephalitis virus (MVEV), St. Louis encephalitis virus (SLEV) are considered to be important pathogens, responsible for significant human and animal disease and mortality. The World Health Organization estimated annual human cases of more than 50 million, 200,000, and 50,000, for DENV [2], YFV [3], and JEV [4], respectively. Severe manifestations of flavivirus disease include, hemorrhaging fever (for YFV and DENV), and encephalitis and neurological sequelae (for JEV, TBEV, WNV, SLEV, and MVEV). Generally, the most lethal flaviviruses, JEV, YFV, TBEV, and DENV, have mortality rates that can range from 5% to 30% [18]. There is no specific therapy available for any flavivirus infection, and currently, commercially available vaccines for human use exist for only three flaviviruses. Additionally, control of mosquito and tick populations has proven difficult [5]. Thus, prevention and treatment of flavivirus disease remain a global public health priority, and extensive efforts have been made toward the development of vaccines and the discovery of potent therapeutic compounds against the most medically important flaviviruses. In this article, current progress in flavivirus vaccine development and drug discovery reported in literature and patents will be reviewed, and the strategies used in these investigations are highlighted.

FLAVIVIRUS GENOME STRUCTURE AND PROTEINS

Flaviviruses are small (50 nm in diameter), enveloped plus-strand RNA viruses Fig. (1A). The virus particle

consists of a nucleocapsid ~30 nm in diameter (containing viral capsid protein and genomic RNA), surrounded by a lipid bilayer in which viral membrane and envelope proteins are embedded. The RNA genome is approximately 11,000 nucleotides (nt) in length and contains an open reading frame encoding a single polyprotein precursor Fig. (1B) [6]. The 5' and 3' untranslated regions (UTRs) are approximately 90-130 and 430-760 nt long, respectively [7]. Both of these UTRs contain RNA elements that are essential for the efficient translation and replication of the flavivirus genome [8]. The 5' end of the genome contains a type-1 cap structure (m⁷GpppA^mp), and the 3' end terminates with a CU_{OH} instead of a poly(A) tail [6]. Translation of the flavivirus genome results in the production of a polyprotein precursor (~3,400 amino acids in length). The polyprotein is co- and post-translationally processed by viral and host cellular proteases, to produce 10 mature viral proteins: capsid (C), premembrane (prM)/membrane (M), envelope (E), and seven nonstructural (NS) proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5. The C, M, and E proteins are structural proteins that comprise the virus particle, whereas the NS proteins are required for genome replication and expression, and may also function in viral assembly, release, and evading immune response [6, 9-15].

FLAVIVIRUS REPRODUCTIVE CYCLE

Flaviviruses enter host cells by receptor-mediated endocytosis Fig. (2) [16]. Low pH triggers fusion between the viral membrane and the host endosomal vesicle membrane, thereby releasing the nucleoplasmid into the cytosol. After disassembly, the viral genome is released and translated into a single polyprotein by the host's translational machinery. The polyprotein is cleaved by host (signalase) and viral (NS2A/NS3) proteases to produced mature viral proteins [17]. The N-termini of PrM, E, NS1, and NS4B are generated by signalase cleavages in the lumen of the endoplasmic reticulum, whereas the majority of the C-termini are generated by cleavage mediated by the NS2A/NS3 protease [8]. Viral replication is catalyzed by a replication complex (composed of NS5, the RNA-dependent

*Address correspondence to this author at the 120 New Scotland Avenue, Wadsworth Center, New York State Department of Health, Albany, New York 12208, USA; Tel: 518-473-7487; Fax: 518-402-4773; E-mail: ship@wadsworth.org

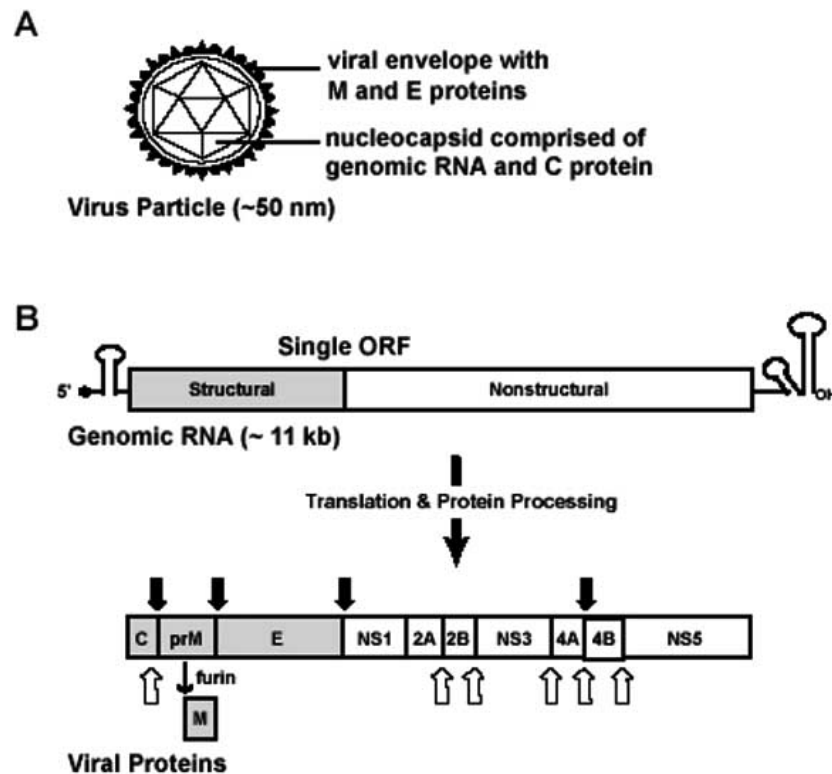


Fig. (1). Virion and genome structure of flaviviruses. (A) The nucleocapsid (genomic RNA within a viral capsid protein shell) is surrounded by a lipid bilayer that contains viral proteins M (solid black ovals) and E (solid black triangles). (B) The flavivirus genome is ~11 kb in length and is capped at its 5' end and has no 3' poly(A)-tail. The stem-loop structures (solid black lines) represent RNA structures present in the 5' and 3' UTRs. Viral structural (gray rectangles) and nonstructural genes (white rectangles) encoded in the (+)-strand RNA genome are also depicted. Processing of the polyprotein by a host signalase (black arrows) or by the viral NS2A/NS3 protease (white arrows) yields the viral proteins (C, prM, E, and NS1-5). A host furin further processes the prM protein to M, which is present in the mature flavivirus virion.

RNA polymerase, and other viral and host factors), which transcribes the plus-strand genomic RNA into a complementary minus-strand RNA. The (-)-strand is then used as a template for the production of progeny (+)-strand genomes [8]. After replication, the viral genome is encapsidated by C and is then directed to the endoplasmic reticulum where the nucleocapsid is enveloped by a lipid bilayer in which viral prM and E proteins are embedded. Immature virions, composed of genomic RNA, prM-E heterodimers, and C, are transported to the cell surface by exocytosis. During this process, prM, E, and NS1 are glycosylated. The prM protein is cleaved by cellular furin to produce glycosylated M protein. PrM processing destabilizes the prM-E interaction and promotes the formation of E homodimers present in mature infectious virions; these are subsequently released extracellularly [17].

FLAVIVIRUS VACCINES

As there are no antiviral drugs or specific therapies against flavivirus infections, vaccination remains the most effective route for controlling these diseases [1]. Currently, human vaccines are available only for YFV, TBEV, and JEV. Yet, despite ongoing vaccination efforts, over 40,000 cases of infection resulting from these three flaviviruses are reported annually [18]. For other human pathogenic flaviviruses including DENV, WNV, SLEV, and MVEV, there are no licensed vaccines available. This is a particularly

important area of research, due to the sheer number of DENV-related illnesses (more than 50 million human cases of DENV infection reported annually) and the rapid spread of WNV in North America alone (>10,000 cases in the US since 1999) [18].

ATTENUATED FLAVIVIRUS VACCINES

Live attenuated viruses are the most successful viral vaccines; approximately 63% of the vaccines approved for use by the US Food and Drug Administration are live attenuated vaccines [19]. This is not surprising, as the immune response to attenuated virus vaccines is broad (since all virally expressed proteins are potential antigens) and long-term, due to their replication capability after vaccination. Two commercially available vaccines are generated from live attenuated flaviviruses [YFV (YF-Vax), and JEV (strain SA14-14-2; licensed in China)], whereas there are three derived from formalin-inactivated whole flaviviruses (JEV (BIKEN), TBEV, and WNV (West Nile Innovator horse vaccine)) [18]. Traditionally, the approach for generation of live attenuated viruses is through empirical passaging of virus in pathogen-free cell culture. More recently, with the advances in recombinant DNA technologies, reverse-genetics systems have been established for many flaviviruses. Thus, it has become possible to engineer attenuated viruses either directly, by introducing stable mutations into an infectious flavivirus cDNA clone, or

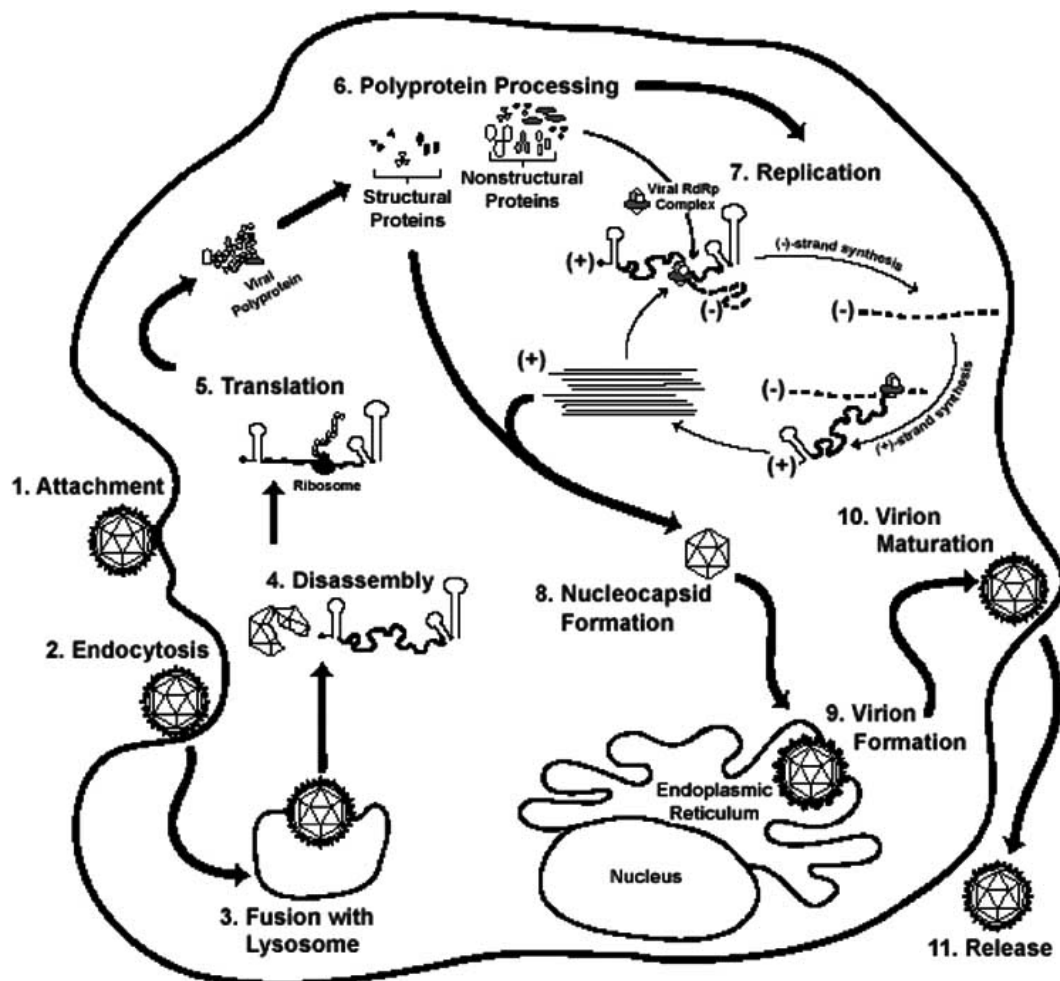


Fig. (2). Flavivirus life cycle. Virions are (1) adsorbed onto host cellular membranes and then (2) enter the cell by receptor-mediated endocytosis. (3) Fusion with lysosomes releases the nucleocapsid, which (4) disassembles to release the capped viral genomic RNA. (5) Translation and (6) polyprotein processing of the flavivirus polyprotein generate individual viral proteins that either participate in viral (7) replication (NS proteins) or (8) nucleocapsid formation (capsid proteins), or have other functions. Progeny (+)-strand viral RNA is generated from a (-)-strand RNA intermediate (dashed line). (9) Virus particles form following virion budding from the endoplasmic reticulum (10). Host furin cleaves prM to generate a mature virion in the endoplasmic reticulum and Golgi complex. The virion is released following vesicle fusion (11). It should be noted that flavivirus translation, replication, and virion assembly occur at the endoplasmic reticulum membrane.

indirectly, by improving pre-existing attenuated flavivirus strains.

It has been recently reported that an attenuated WNV (WN1415) of the non-epidemic strain (lineage 2) can be used as an effective vaccine against the virulent epidemic strain (lineage 1) in mice [20]. The attenuated phenotype of the WN1415 vaccine candidate likely results from the many spontaneous mutations within this strain's genome relative to the progenitor wild-type strain, the most prominent of which is a 76 nt deletion in the 3' UTR. Although the mechanism of the attenuation remains to be characterized, mutagenesis studies with a WNV subgenomic replicon (containing a deletion of viral structural genes) showed that the 3' UTR regulates viral replication [21-23]. Interestingly, mutagenesis (in the 3' UTR) has also been used to engineer attenuated strains of DENV and TBEV [24, 25]. In DENV-1, mutations were introduced into the lower part of the 3' stem-loop structure of the genome, to attenuate virus replication in

monkeys. This mutant virus (DENV1mutF) was highly immunogenic and protective against viral challenge [25, 26]. For DENV-4, attenuation was achieved by introducing a 30-nt deletion into the 3' UTR; this resulted in a monovalent vaccine (rDEN4 30) that is safe, as determined by phase I and II clinical trials [27]. It is generally accepted, however, that a successful DENV vaccine candidate must protect against all four DENV serotypes, as a tetravalent vaccine. For development of the DENV tetravalent vaccine, the 30 nt 3' UTR deletion was introduced into the other three DENV serotypes (1 to 3) along with: (i) a panel of substitutions to further attenuate the viruses; and (ii) adaptative mutations to improve virus growth in Vero cells. Additionally, the prM/E from DENV-3 was subsequently cloned into a DENV-4 backbone since the DENV-3 30 nt 3' UTR mutant was underattenuated in monkeys [28-30]. Two doses of two different vaccine formulations (TV-2 and TV-3) induced high balanced neutralizing antibody titers,

broad neutralization activity, and protection against DENV challenge in rhesus monkeys. Further studies aim to improve these tetravalent vaccines because interference between the different attenuated DENV viruses was observed. Similarly, interference has been observed for a tetravalent DENV vaccine generated from passage in PDK cells [31, 32]. Currently, tetravalent formulations and immunization schedules are being optimized, so as to confer similar levels of protection against all four DENV serotypes [33].

A number of flavivirus NS proteins were recently shown to suppress host antiviral immune response. Expression of DENV NS4B and, to a lesser extent, NS2A and NS4A, results in down-regulation of interferon (IFN)- α -stimulated gene expression [15]. More recently, it was found that the N-terminal 125 amino acids of DENV NS4B are responsible for inhibition of IFN signaling [34]. JEV infection, like DENV infection, blocks the IFN- α -induced Jak-Stat signaling pathway [35]. During WNV infection, host response was found to limit viral spread through the activation of the IFN regulatory factor 3 pathway [36]. IFN- α -signaling and STAT2 translocation to the nucleus were inhibited when Kunjin (a subtype of WNV occurring in Australia) NS2A, NS2B, NS3, NS4A, and NS4B, but not NS1 and NS5, were individually expressed [11]. Furthermore, WNV replication was shown to prevent the phosphorylation and activation of the Janus kinase JAK1 and Tyk2 [12]. Although the molecular details of how the NS proteins inhibit host immune responses remain to be determined, this information has provided a novel strategy for the development of flavivirus vaccines. Mutation(s) to weaken or knock out IFN inhibition could be introduced into a flavivirus infectious cDNA clone, to generate attenuated viruses that are replication-competent, but defective in IFN antagonism. Upon inoculation of animals, the mutant virus will replicate and elicit a strong immune response. Since the virus is sensitive to the host's IFN system, the virus will be eliminated by the host without causing disease.

CHIMERIC FLAVIVIRUS VACCINES

Derived from the attenuated YFV Asibi strain, YFV 17D is one of the most highly efficacious viral vaccines to have been developed, thus far [5]. This vaccine is both safe and highly immunogenic, and it is thought to confer a life-long immunity to YFV after a single dose [18]. As outlined in a patent [37], several chimeric flavivirus vaccines take advantage of a YFV 17D viral vector (which expresses YFV C and NS proteins) to deliver heterologous flavivirus prM and E proteins. Such vaccines (ChimeriVax-WNV, ChimeriVax-JE, and ChimeriVax-DEN, all from Acambis) have been developed and are in clinical trials. For WNV, a second-generation vaccine (ChimeriVax-WNV₀₂) has been engineered [38]. Three point mutations were introduced into the E protein: two to further attenuate the virus, and one to reduce its neuroinvasiveness in mice. Vaccination of monkeys with ChimeriVax-WNV₀₂ conferred solid protection against lethal wild-type WNV challenge. In JEV, the prM/E genes from the attenuated SA14-14-2 vaccine strain was used to replace corresponding genes in YFV 17D. Interestingly, the three E protein substitutions introduced into ChimeriVax-WNV₀₂ are also present in the E protein of ChimeriVax-JE. Clinical phase II trials indicate that

ChimeriVax-JE is safe and able to induce protective titers of neutralizing antibodies in humans [39]. Additionally, antisera raised from mice immunized with ChimeriVax-JE are able to protect naïve mice from other genotypes of JEV (strong protection against genotypes II and III, lesser protection against genotypes I and IV) [40]. Patented technologies [41] have been utilized in the development of tetravalent formulations of chimeric YFV-DEN vaccines (ChimeriVax-DEN1-4), which were tested in monkey models. Similar to the other ChimeriVax vaccines, the tetravalent DENV version does not produce serious adverse effects, and has lower neurovirulence and hepatotropism than does a YFV 17D control. Monkeys were protected from DENV challenge (100% from DENV2 and DENV3; 83% for DENV1 and DENV4) 6 months after a single immunization [42, 43]. Another candidate vaccine, D2/WN, replaces DENV prM/E genes with those of WNV. This attenuated chimeric virus is immunogenic and induces protective ability against WNV in mice. The DENV PDK-53 backbone has been shown to be safe and effective in human clinical trials [44]. Other patented technologies have described similar approaches to generate chimeric DENV virus vaccine by introducing WNV or TBEV prM/E genes into a DENV virus backbone [45, 46]. The WNV and TBEV vaccine candidates were highly immunogenic and conferred immunity against virus challenge.

HETEROLOGOUS VIRUS VACCINES

Two promising systems have been recently utilized for the development of flavivirus vaccines. Both exploit heterologous virus vaccine vectors to express flavivirus antigens. The first system takes advantage of a recombinant avian virus vector (canarypox virus) engineered to express WNV prM and E genes [47]. The canarypox backbone vector (ALVAC) is attenuated, considered safe, and does not replicate in non-avian hosts [48]. Immunization with the canarypox-WNV vaccine induced virus neutralizing antibodies in dogs, cats, and horses, and it conferred protection against viremia [49]. Currently, this vaccine is licensed (Merial) and is available for use on equids [50].

The second system utilizes a live attenuated measles virus (MV Schwarz strain) to express WNV prM/E genes. This attenuated measles virus is one of the safest, most stable, and most effective human vaccines [51]. The MV-WNV vaccine induces high levels of neutralizing antibodies and confers protection from a lethal dose of WNV in mice. Furthermore, this vaccine expresses foreign proteins in the presence of pre-existing immunity to MV [52]. Both canarypox/WNV and MV/WNV heterologous viral vaccines have shown promise; however, further clinical evaluation will be needed to determine the efficacy of these vaccines in non-human primates and in humans. Others have developed a modified vaccinia virus Ankara strain to express DENV prM/E/NS1 antigens. These studies are in their preliminary stages, as no immunological data in cell culture or animal systems have been presented [53].

FLAVIVIRUS DNA VACCINES

DNA-based strategy has also been explored for development of flavivirus vaccines [54, 55]. Viral immunization using DNA vaccines involves the direct

inoculation of animals with purified plasmid DNA (using syringe or needle-free systems), which facilitates uptake of the DNA by cells, and allows the expression of plasmid-encoded viral antigens that induce immune responses (both cytotoxic and humoral) protective against viral infection. Several studies have demonstrated that direct inoculation of DNA expression plasmids (encoding flavivirus antigens) can confer immunity to flavivirus infection in mice [56-60], and partial protection in monkeys [61-64]. The success of this approach correlates with the ability of the prM and E proteins to assemble nucleocapsid-free virus-like particles (VLPs). These VLPs are secreted from cells and can elicit a strong immune response; they therefore are able to induce protection against flavivirus challenge [60, 65-67]. DNA vaccines encoding the flavivirus gene NS1 have also been shown to protect mice from flavivirus challenge [68-70]. Additionally, a DNA vaccine containing the cDNA of an attenuated (by Leu250 to Pro mutation in NS1) Kunjin virus replicon was shown to induce protective immune responses in mice [71]. Vaccination of mice with 0.1-1 µg of a full-length infectious DNA plasmid of Kunjin virus was able to confer solid protection against lethal challenge with the virulent New York strain of WNV [72].

Recent efforts in flavivirus DNA vaccine research have focused on increasing the immunogenicity of plasmid-expressed flavivirus antigen. Optimization of vaccine delivery, through injection of DNA absorbed onto cation microparticles, *in vivo* electroporation, or needle-free (gene gun) delivery systems, has been reported to enhance immunogenicity, by increasing the transfection efficiency of cells with DNA vaccines [56, 73, 74]. Another approach is to co-express foreign genes and sequences with viral antigen so as to modulate immune response. In DENV-1, co-administration of plasmids (using a needle-free system) encoding prM and E antigens as well as human immunostimulatory sequences and a cytokine (granulocyte macrophage colony stimulating factor) significantly improves the strength and duration of protection observed in virus-challenged monkeys, as compared to DNA vaccines alone [75]. Furthermore, for DENV-2 and WNV, DNA vaccines encoding a chimeric prM/E/lysozyme-associated membrane protein specifically target the viral antigen to the major histocompatibility compartment (MHCII) of antigen-presenting cells, and induce significantly higher antibody levels and long-term neutralization titers, in comparison to DNA vaccines expressing untargeted flavivirus antigen [76, 77]. Although these results are encouraging, further studies are needed to precisely determine which approaches will be safest and most efficacious, while remaining feasible for human use. In April 2005, it was announced that a human WNV DNA vaccine (expressing WNV prM and E proteins with a JEV signal sequence to optimize VLP secretion) is in Phase I clinical trials launched by the National Institute of Allergy and Infectious Disease in collaboration with Vical Inc. (the company who has a nonexclusive license from the Center for Disease Control) [54]. This vaccine has been shown to confer WNV immunity to mice, horses, and birds [56, 78].

FLAVIVIRUS RNA VACCINES

Another promising immunization approach is the direct use of mutant viral and replicon RNA. For flaviviruses, studies have described the direct inoculation of *in vitro* transcribed flavivirus RNA from attenuated infectious (470 nt deletion in the 3' UTR) and non-infectious [61 amino acid deletion in the C protein) TBEV mutants, to immunize mice against lethal doses of wild-type TBEV [79, 80]. Both approaches are suggested to exploit the ability of these viral RNAs to (i) form and release highly immunogenic viral particles (infectious for the 3' UTR mutant, and non-infectious VLPs for the C mutant); and (ii) replicate *in vivo*, and therefore express all viral NS proteins so as to elicit a comprehensive immune response resembling that of conventional live virus vaccines. The C mutant is particularly interesting, due to its safety (it cannot produce infectious viral particles and therefore is unable to spread within the host) and its ability to elicit highly titered and highly protective immune responses in mice.

FLAVIVIRUS SUBUNIT VACCINES

Direct vaccination with purified protein subunits can elicit an immune response in many animals. In flaviviruses, the E protein is the most commonly used antigenic protein. This is because the E protein is highly immunogenic and elicits a strong and long-lasting immune response [81]. In WNV, a truncated E protein expressed and purified from *Drosophila* S2 cells was suggested to have a conformation similar to that of native full-length E protein. When used in a subunit vaccine, the recombinant E protein was able to protect mice from a lethal dose of WNV [82]. In JEV, E protein purified as VLPs from a stable cell line was able to confer complete protection against challenge from the wild-type virus [83]. When an alternative yeast-based purification approach was utilized, purified DENV recombinant E protein could only elicit partial protection against DENV-4 challenge in monkeys [84]. In this instance, the E antigen may have not been sufficiently purified (70% purity) or it may not have been as antigenic as expected due to protein misfolding. Interestingly, in JEV, smaller subfragments of E (N-terminal 300 amino acids) were found to be as effective as a commercially available vaccine, when boosted (1 week after the initial protein immunization) with a DNA vaccine expressing the same E fragment [85].

FLAVIVIRUS ANTIVIRALS

Identification of anti-flavivirus inhibitors is critical to the development of therapeutic treatments directed against flavivirus disease. Potentially, these anti-flavivirus agents could target/inhibit any process critical for virus reproduction, refer to Fig. (2). Many approaches are being utilized to identify potential antiviral chemotherapeutic compounds, including: (i) rational design based on the 3D crystal structures of viral proteins or secondary/tertiary structures of viral genomic RNA; (ii) screening of large compound libraries of potential antiviral agents; (iii) testing of known inhibitors of other viruses; (iv) chemical modification of known viral inhibitors in order to optimize their function; and (v) intravenous immunoglobulin- and nucleic acid-based

therapy. Some of these approaches have yielded anti-flavivirus compounds that may be of therapeutic use.

INTRAVENOUS IMMUNOGLOBULIN-BASED THERAPY

Passive transfer of intravenous immunoglobulin is being investigated for the treatment of flavivirus infection. Pre-administration of serum or pooled plasma (from WNV infected mice) or polyclonal human gamma globulin (from WNV infected humans) protected mice from WNV infection. Post-administration of these treatments offered WNV challenged mice partial protection, in a dose-dependent manner [86, 87]. It is interesting to note that a 20-35% improvement in mortality rate and survival time was observed after human gamma globulin administration 5 days following WNV infection, when WNV is uniformly disseminated to the mouse brain [86]. Strikingly, a humanized monoclonal antibody (E16) against domain III of the E protein administered to mice 5 day after exposure to WNV improved survival to 90%. Viral plaque assays demonstrated that WNV was completely cleared from the brains of 68% of the surviving mice [88]. These experiments in mice underscore the broad utility of humanized antibodies as a post-exposure therapy for flavivirus infection.

NUCLEIC ACID-BASED THERAPY

Nucleic acid-based approaches have been explored for flavivirus therapy, including antisense-, ribozyme- and RNA interference (RNAi)-based technologies. RNA silencing (siRNA) was shown to suppress DEN-2 [89] and WNV [90] in tissue culture. Advances in antisense technology, namely, (i) the development of phosphorodiamidate morpholino oligomers (PMOs) [91], and (ii) the conjugation of these PMOs with arginine-rich peptides [92], have been combined to create a novel approach to the inhibition of flavivirus replication in cells [93]. These specialized antisense oligomers are efficiently taken up by cells; they are nuclease-resistant; they bind to RNA; and their antiviral activities are not due to cytotoxicity. Recent studies in WNV and DENV have taken advantage of these arginine-rich antisense PMOs to target RNA elements critical for flavivirus reproduction [94, 95]. The most effective PMOs those complementary to viral 5' ends and 3' CS (a *cis*-RNA element essential for flavivirus replication) reduced viral levels by 5-6 logs. Interestingly, in WNV, the 5' and 3' CS PMOs were shown to specifically inhibit viral translation and replication, respectively [95]. Future studies should address whether these compounds are effective therapeutic agents against flavivirus infection in animal models.

PROTEASE / HELICASE INHIBITORS

Inhibition of the helicase and protease activities of flavivirus NS3 has long been an attractive target for antiviral drug discovery, since these activities are essential for productive flavivirus infection. Previous studies have reported that a nucleoside analogue (HMC-HO4) inhibits *in vitro* WNV helicase activity and WNV replication in Vero cells, at an IC₅₀ of ~30 μM [96]. More recently, other small flavivirus-specific helicase inhibitors, such as certain ring-expanded nucleoside analogues, and halogenated benzimidazoles and benzotriazoles, have also been shown to

inhibit WNV NS3-mediated unwinding of RNA substrates at low concentrations (IC₅₀ 0.3-15 μM). This effect is specific for viral helicase; the activity of a cloned human NTPase/helicase or viral NTPase is not inhibited by these compounds [97-99]. Although comprehensive cytotoxicity assays have yet to be performed, one study reports that the cytotoxicity of at least one of these halogenated compounds (4,5,6,7-tetrabromo-1H-benzotriazole: TBBT) can be improved after N-alkylation [100].

The flavivirus proteases, unlike host cellular proteases, recognize cleavage sites containing dibasic amino acid residues (at positions P1 and P2) and a small amino acid side chain (position P1'). Thus, it is not surprising that conventional cellular protease inhibitors such as benzamidine and PMSF are inactive against DENV and WNV NS3 proteases [101, 102]. Recently, compounds capable of inhibiting the protease activity of NS3 have been identified. Some are short peptides that mimic the protease cleavage site, and thus competitively inhibit NS3 protease activity by binding to its catalytic site [101, 103]. This hypothesis is supported by modeling studies, which indicate that the active site of DENV NS3 (S1 pocket residues Asp 129, Ser 135, Tyr 150, and Ser 163) interacts with the P1 substrate (Arg or Lys) [104, 105]. In another study, using crystallography-based models for NS3-substrate interaction, compounds containing single guanidino groups that could potentially interact with amino acids in the S1 pocket of NS3 were identified [103]. Furthermore, these modeling studies suggest that compounds containing a single guanidino arm inhibited WNV and DENV NS3 protease activity (with K_i values at less than 23 μM), by forming hydrogen bonds with the active Ser135 residue in the S1 pocket. Although several compounds can effectively inhibit the *in vitro* helicase or protease activity of NS3, future research should aim to improve their specificity and potency, and should address their antiviral activity in cell cultures and animal models.

NUCLEOSIDE ANALOGS

Virally encoded RNA-dependent RNA polymerases of RNA viruses, because they are essential for the replication of the RNA viral genome, are also attractive targets for antiviral agents. Nucleoside analogs have been well characterized and are approved therapeutics for treatment of viral infection by HIV and hepatitis B, for example. Recent reports have indicated that a modified nucleoside -D-CH₃-riboA can inhibit YFV (EC₅₀ = 0.2 μM) in a plaque reduction assay [106]. Additionally, adenosine substituted with methyl groups at the 2'-C position can effectively inhibit (by chain-termination of RNA synthesis) DENV, WNV, and YFV in Vero cells, at EC₅₀ values of 4 μM, 5.1 μM, and 3.2 μM, respectively [107]. Modification of the 2'-C-adenosine compound to a 7-deaza 2'-C-methyl-adenosine decreased its cytotoxicity to a CC₅₀ >1mM in Jurkat cells [108]. When used as antiviral drugs, these nucleosides can be incorporated into cellular RNAs. However, the level of incorporation is reduced, compared to the therapeutic anti-HIV acyclovir control. It is important to emphasize that the 7-deaza 2'-C-methyl-adenosine compound is (i) more specific and potent for HCV; and (ii) a single point mutation within the HCV NS5B polymerase active site (S282T) confers insensitivity to the nucleoside analogue [108].

Hence, for flavivirus systems, further optimizations using medicinal chemistry may be required, to increase the efficacy of these inhibitors and to limit the possibility of generating flavivirus escape mutants. Besides direct incorporation into RNA chain to block viral replication (described under this section), some other nucleoside analogs exert their antiviral activities through inhibition of nucleoside triphosphate synthesis in host cells (see below).

VIRAL ENTRY INHIBITORS

Several inhibitors implicated in flavivirus entry into the host have recently been reported. These antiviral compounds can be categorized as sulfated polysaccharides and polyoxotungstates. Six polyoxotungstates were able to inhibit DENV-2, at IC_{50} values of 0.45-36.8 μ M, without any apparent cytotoxic effects in Vero cells [109]. Two vanadyl-substituted polyoxotungstates had the most potent antiviral activity (IC_{50} 0.45-1.95 μ M). Further analysis of one of the vanadyl-substituted compounds indicated that, for HIV, binding of virions to cell membranes was affected. Although a similar function was implied, no specific mode of action analysis was conducted for DENV-2. In contrast, the activity of sulfated polysaccharides have been examined in more detail; these compounds have been suggested to inhibit DENV-2 (IC_{50} \sim 1.0 μ g/ml) adsorption and internalization in human and monkey cells, but not in mosquito cells [110, 111]. Two specific compounds (G3d and C2S-3) inhibited DENV-3 (IC_{50} 13.9-14.2 μ g/ml), and to a lesser degree DENV-1 and DENV-4 (IC_{50} 29.3 μ g/ml to $>$ 50 μ g/ml). Other sulfated polysaccharides (sulfated galactomannans) protected mice from symptoms of disease and death when co-inoculated with lethal doses of YFV; however, no protection was afforded when these compounds were administered 3 days post-infection [112]. It was suggested that the sulfated galactomannans inhibit either entry or an early step (pre-translation) in viral reproduction. Alternatively, these compounds may have virucidal properties. Further studies are required to differentiate between these two modes of action.

INHIBITORS OF FLAVIVIRUS ASSEMBLY

Earlier reports have described compounds, such as N-nonyl-deoxyojirimycin and 6 O-butanoyl castanospermine, that inhibit DENV-2 and JEV through the inhibition of cellular glycoprotein processing α -glucosidase enzymes [113, 114]. These inhibitors strongly affect N-linked oligosaccharide trimming of prM, E, and NS1, leading to protein misfolding, reduction in glycoprotein secretion, and decreased intracellular levels of NS1. The former two defects contribute to an unproductive virion assembly pathway whereas the latter is suggested to reduce virus replication. In agreement with above studies, castanospermine was recently shown to be a potent inhibitor of dengue virus infection *in vitro* and *in vivo* [115].

INHIBITORS OF NUCLEOSIDE TRIPHOSPHATE SYNTHESIS

Several inhibitors of nucleotide triphosphate synthesis (orotidine monophosphate decarboxylase (OMPDC), inosine monophosphate dehydrogenase (IMPDH), and CTP synthesis) have been recently reported to have antiviral

properties against WNV [116]. These compounds, which include mycophenolic acid (Therapeutic Index, or TI=71), 6-azauridine (TI=131), 6-azauridine acetate (TI=62), pyrazofurin (TI=18), 2-thio-azauridine (TI=125), and cyclopentenylcytosine (TI=71), were found to be the most active, among a panel of 34 compounds tested. Ribavirin was also shown to have anti-WNV activity, through at a lower potency (TI $>$ 9.4). All of the compounds inhibit OMPDC except for mycophenolic acid and ribavirin (which inhibit IMPDH) and cyclopentenylcytosine (which inhibits CTP synthesis). Besides inhibition of IMPDH, ribavirin was reported to have other antiviral mechanisms such as functioning as a mutagen to cause error catastrophe or serving as a RNA cap analog [117, 118]. Mycophenolic acid appears to be the most promising of these inhibitors, due to its potency at therapeutic concentrations and demonstrated antiviral activity for other flaviviruses [117, 118]; however, successful *in vivo* efficacy results have not yet been reported. Mechanistically, mycophenolic acid was shown to directly bind IMPDH [119], and decrease guanosine levels to a point where viral RNA synthesis is adversely affected [120]. One patent indicates that mycophenolic acid has undesirable pharmacological properties and describes, as an alternative, carbamate prodrugs that produce active IMPDH inhibitors *in vivo* [121]. Potentially, these prodrugs could be screened for antiflavivirus activity or, could be used to improve mycophenolic acid or other nucleoside triphosphate inhibitors by chemical modification.

OTHER ANTIVIRAL COMPOUNDS

Brequinar is an antiviral drug that functions as an inhibitor of cellular dihydroorotate dehydrogenase. Brequinar and other dihydroorotate dehydrogenase inhibitors were shown to be potent inhibitors of YFV, Kunjin virus, and DENV, in Vero and human cells [122]. These compounds have potent antiviral activities (for example, EC_{50} of 0.02 μ M and TI of 2600 for Brequinar). However, *in vivo* efficacy of the compounds remains to be determined.

IFN- β and diethylthiocarbamate (DDTC) are two potential therapeutic compounds that are safe during clinical use [123, 124]. DDTC delays morbidity; it protected 25% of mice from a lethal dose of JEV [125]. This compound is thought to inhibit JEV infection through its ability to cross the blood-brain barrier [126] and to enhance macrophage and cytotoxic killer T-cell activity [127]. IFN- β was shown to be therapeutic when applied to cells infected with WNV [124]; it was able to reduce morbidity, mortality, and viral load (by 80-100%) in immunocompromised mice infected by Modoc flavivirus. Moreover, in a nonrandom preliminary trial during a human SLEV outbreak, IFN- β reduced the disease symptom severity and duration [128]. In another instance, three individuals displaying multiple WNV-mediated neurological symptoms were treated with IFN- β . All three showed marked improvement (one patient recovered), thereby encouraging further evaluation of this compound for treatment of flavivirus infection [129].

CURRENT AND FUTURE DEVELOPMENTS

There are several promising vaccine candidates and antiviral compounds that have demonstrated antiflavivirus activity. Currently, some of the vaccine candidates are

undergoing clinical trials, to determine their safety and efficacy in humans. Furthermore, as most clinical vaccination trials are carried out in naïve subjects, it will be important to extend these studies to determine whether individuals previously infected with flavivirus can be effectively and safely vaccinated. The YFV 17D-based chimeric vaccines showed great promise. For example, YFV 17D-based JEV vaccine has successfully gone through phase I and II clinical trials. However, it should be noted that, despite the potential success of this approach, other vaccine strategies (including attenuated virus-based approaches and recombinant protein-based immunization) are still worth investigating. For antiviral discovery, it is critical to develop high-throughput screening (HTS) assays. Novel HTS assays could be developed by engineering a reporting gene (luciferase or green fluorescent protein) into the reverse-genetic systems of flaviviruses. In the case of WNV, three genetics-based HTS assays have been established: (i) a cell line harboring persistently replicating subgenomic replicon, (ii) packaged VLPs containing replicon RNA, and (iii) a full-length reporting virus. For each assay, a luciferase gene was engineered into the replicon or into the full-length viral genome, to monitor viral replication. Potential inhibitors could be identified through suppression of luciferase signals upon compound incubation [95, 130]. These assays allow for the screening of potential inhibitors of viral replication as well as viral entry and virion assembly. The latter two aspects of the assays are important for flavivirus drug discovery, because recent structural studies showed that, in addition to viral replication, flavivirus entry and assembly are attractive antiviral targets. In addition, because each of the above assays encompasses multiple, but discrete, steps of the viral life cycle, these assays could potentially be used to discriminate the mode of action of any inhibitor, whether effecting viral entry (detected by assays ii and iii, but not by assay i), replication (including viral translation and RNA synthesis; detected by assays i - iii), or virion assembly (detected by assay iii, but not by assays i and ii). Furthermore, a transient reporting replicon system of WNV has been developed to determine the mode of action of inhibitors, and to differentiate between those that inhibit viral translation and those that inhibit RNA synthesis [131, 132]. The approaches described for WNV should be applicable to development of cell-based assays for other flaviviruses. The establishment of these HTS assays will greatly facilitate flavivirus drug discovery.

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